

University of Groningen

Potential targets for immunotherapy and infection imaging on the cell surface of *Staphylococcus aureus*

Romero Pastrana, Francisco

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version

Publisher's PDF, also known as Version of record

Publication date:
2017

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Romero Pastrana, F. (2017). *Potential targets for immunotherapy and infection imaging on the cell surface of Staphylococcus aureus*. [Thesis fully internal (DIV), University of Groningen]. University of Groningen.

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Chapter 4

A human monoclonal antibody that specifically binds and inhibits the staphylococcal complement inhibitor protein SCIN

Hedzer Hoekstra, Francisco Romero Pastrana, Hendrik P. J. Bonarius, Kok P. M. van Kessel, Goffe S. Elsinga, Neeltje Kooi, Herman Groen, Jan Maarten van Dijl and Girbe Buist

Published in *Virulence*. 2017; 0:1–13

Abstract

Staphylococcus aureus is a serious public health burden causing a wide variety of infections. Earlier detection of such infections could result in faster and more directed therapies that also prevent resistance development. Human monoclonal antibodies (humAbs) are promising tools for diagnosis and therapy owing to their relatively straightforward synthesis, long history of safe clinical use and high target specificity. Here we show that the humAb 6D4, which was obtained from a random screen of B-cells producing antibodies that bind to whole cells of *S. aureus*, targets the staphylococcal complement inhibitor (SCIN). The epitope recognized by 6D4 was localized to residues 26 to 36 in the N-terminus of SCIN, which overlap with the active site. Accordingly, 6D4 can inhibit SCIN activity as demonstrated through the analysis of C3b deposition on *S. aureus* cells and complement-induced lysis of rabbit erythrocytes. Importantly, while SCIN is generally regarded as a secreted virulence factor, 6D4 allowed detection of strongly increased SCIN binding to *S. aureus* cells upon exposure to human serum, relating to the known binding of SCIN to C3 convertases deposited on the staphylococcal cell surface. Lastly, we show that labelling of humAb 6D4 with a near-infrared fluorophore allows one-step detection of SCIN-producing *S. aureus* cells. Together, our findings show that the newly described humAb 6D4 specifically recognizes *S. aureus* SCIN, which can potentially be used for detection of human serum-incubated *S. aureus* strains expressing SCIN.

Introduction

Staphylococcus aureus is a highly adaptable and dangerous Gram-positive bacterial pathogen that is asymptotically carried by about one-third of the human population. *S. aureus* can cause a wide variety of infections due to its extensive arsenal of virulence factors¹. A subset of these virulence factors target the human immune system by blocking chemotaxis of phagocytes, complement activation, oxidative killing or phagocytic uptake. Alternatively, they may redirect host defenses, such as fibrin formation or formation of neutrophil extracellular traps to favor pathogen replication². Thus, the response of *S. aureus* to the human immune system is highly flexible, allowing survival in the host's hostile environment³. Due to its adaptability *S. aureus* has also become resistant to a broad spectrum of antibiotics⁴, and nowadays the drug-resistant lineages of *S. aureus* represent a serious public health burden^{2,5}. This applies in particular to methicillin-resistant *S. aureus* (MRSA), which causes significantly increased morbidity and mortality worldwide^{6,7}. Vancomycin has been the drug of choice to treat MRSA infections, but strains have emerged that display reduced vancomycin susceptibility⁸. This implies that there is an urgent need for new and reliable approaches to prevent and treat infections by drug-resistant staphylococci.

Immune therapies against *S. aureus* infections have been explored as a treatment alternative to antibiotics. While active immunization could potentially prevent the onset of *S. aureus* infections, passive immunization could be applied to treat acute or current infections. While the use of pooled human sera does not seem to be very effective^{9,10}, passive immunization with monoclonal antibodies, preferably human monoclonal antibodies (humAbs), is an attractive alternative option. Importantly, humAbs have a high specificity, their synthesis is relatively straightforward, and they have a long history of safe use^{11,12}. However, despite recent successes in animal models^{13–15}, the efficacy of passive immunization with humAbs has not yet been confirmed in clinical trials¹¹.

Wounds of patients with the genetic blistering disease epidermolysis bullosa (EB) are highly susceptible to bacterial colonization¹⁶. In a study by van der Kooi-Pol et al., it was documented that essentially all investigated EB patients with chronic wounds were heavily colonized with *S. aureus*¹⁷. Interestingly, it was noted that these patients did not frequently suffer from *S. aureus* bacteraemia, despite the impaired barrier function of the skin. Compared to healthy individuals, the plasma of EB patients contained significantly higher IgG1 and IgG4 levels, suggesting a potentially protective effect of anti-staphylococcal antibodies against invasive staphylococcal infections^{18,19}. In a recent project, we therefore collected B-cells from donors with EB and applied them to develop a set of fully human monoclonal antibodies against molecules exposed on the cell surface of *S. aureus*^{13–15}. The present study was aimed at the characterization of one of these humAbs referred to as 6D4. In brief, our results show that the humAb 6D4 binds specifically to the staphylococcal complement inhibitor (SCIN), thereby inhibiting its activity. Furthermore, using 6D4, we show that cell surface binding of SCIN is enhanced in the presence of human serum.

Materials and Methods

Strains and growth conditions

Strains used in this study are listed in Table 1. *E. coli* Rosetta Gami (DE3) pLysS strains (Novagen, Merck Biosciences Darmstadt, Germany) carrying prSETB-derived plasmids with the genes encoding for SCIN, OrfD or the respective chimeric constructs have been previously described²⁰. *E. coli* Rosetta gami strains were grown overnight in Lysogeny Broth (LB, Becton Dickinson, Breda, The

Netherlands) at 37°C under vigorous agitation (250 rpm), in the presence of ampicillin (50 µg/ml) and chloramphenicol (34 µg/ml) for plasmid selection. All staphylococcal strains were cultured overnight in TSB (Oxoid Limited, Hampshire, UK) at 37°C under vigorous agitation (250 rpm), unless otherwise specified. *L. lactis* strains were grown at 30°C in M17 broth (Oxoid Limited), or on plates containing 1.5% agar and 0.5% glucose (wt/vol), supplemented with chloramphenicol (5 µg/ml) for plasmid selection.

Sample preparation, SDS/LDS-PAGE, Western blotting and Immunodetection

For the production of chimera of SCIN and the homologous OrfD protein of unknown function overnight cultures of previously described *E. coli* Rosetta gami strains²⁰ were diluted to an optical density at 600nm (OD₆₀₀) of 0.1. Chimeric protein production was induced at an OD₆₀₀ of ~0.5 by the addition of 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG). After 4 h of continued cultivation, cells were collected by centrifugation, and the SCIN-OrfD chimeras produced by these cells were separated by SDS-PAGE as previously described²⁰. The replacement of SCIN residues with corresponding OrfD residues is detailed in Figure 2 and the corresponding legend.

For the preparation of LDS-PAGE samples, *S. aureus* cells collected by centrifugation were disrupted with 0.1 µm glass beads (Biospec Products, Bartlesville, USA) in a Precellys 24 homogenizer (Bertin Technologies, France), and resuspended in LDS sample buffer (Life Technologies). Growth medium fractions were prepared for LDS-PAGE as described before¹. Proteins were separated on NuPAGE gels (Life Technologies) and either visualized by Simply Blue Safe Staining (Life Technologies)¹ or Western blotting using either mouse anti-His tag (Life Technologies), IRDye 800CW-labelled humAb 6D4, or IRDye 800CW-labelled secondary goat anti-human or goat anti-mouse antibodies (LI-COR Biosciences). Bound antibodies were visualized using an Odyssey Infrared Imaging System (LI-COR Biosciences).

Expression of Staphylococcal SCIN and CHIPS proteins in L. lactis

Primers used for cloning are described in Table 2. DNA amplification was carried out using Fusion Hot start High-Fidelity DNA polymerase according the instructions of the supplier (Thermoscientific). Bacterial chromosomal DNA was isolated using the ZR BAC DNA Miniprep Kit (Zymo Research Corporation, USA) following the manufacturer's protocol. Primer pairs Scin-up/Scin-low used for detection of *scn*, the gene encoding SCIN, were used as previously described³¹. Cloning of the PCR-amplified *scn* and *chp* genes was carried out by *Not*I and *Bam*HI (New England Biolabs) cleavage followed by ligation to *Not*I/*Bam*HI cleft plasmid pNG4210³³. Ligated mixtures were used to transform electrocompetent *L. lactis* PA1001 as described³⁴. All constructs thus obtained were verified by sequencing (Eurofins MWG Operon, Ebersberg, Germany).

The production of secreted SCIN and CHIPS in exponentially growing (~0.5 OD₆₀₀) cultures of *L. lactis* was induced by the addition of nisin (3 ng/ml, Sigma-Aldrich, St. Luis, MO). Growth medium fractions were harvested after overnight incubation at 30°C, and proteins in these fractions were analyzed by LDS-PAGE, Simply Blue Safe Staining, or Western blotting as described above.

S. aureus incubation in human sera

Cells of *S. aureus* Newman $\Delta spa \Delta sbi$ were collected from the growth medium by centrifugation at 14.000 rpm for 2 min. The supernatant fraction, containing secreted SCIN, was collected. Next, the collected cells were resuspended and incubated with 20% human serum in HBS (Hepes Buffered Saline; 20mM Hepes, 140 mM NaCl) plus 5 mM CaCl₂ and 2.5 mM MgCl₂ for 30 min to coat the bacteria with C3B and allow for the formation of C3 convertases. Subsequently, the cells were

incubated in PBS at 37°C for 30 min to dissociate surface-bound C2a/Bb. Where appropriate, the collected *S. aureus* supernatant was added to the C3 convertase-covered bacteria to allow binding of SCIN to the surface-attached C3 convertase. The protocol for blood donations from healthy volunteers was approved by the Independent Ethics Committee of the Foundation 'Evaluation of Ethics in Biomedical Research' (Assen, the Netherlands). This protocol is registered by QPS Groningen (code 04132-CS011). The required written consent was obtained for all donors included in the present studies.

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant phenotype(s) or genotype(s)	Reference
<i>S. aureus</i> Newman	NCTC 8178 clinical isolate	21
<i>S. aureus</i> Newman $\Delta spa\Delta sbi$	<i>spa sbi</i> mutant	22
<i>S. aureus</i> USA300	Community-acquired MRSA isolate	23
<i>S. aureus</i> SH1000 $\Delta spa::kan$	<i>rsbU</i> ⁺ , <i>agr</i> ⁺ ; replacement of <i>spa</i> by kanamycin resistance marker (Kan ^R)	1
<i>S. aureus</i> N315	Hospital-acquired MRSA isolate	24
<i>S. aureus</i> NCTC8325 $\Delta pknB$	NCTC8325 (wild-type, 11-bp deletion in <i>rsbU</i>) containing <i>pknB</i> deletion	25
<i>S. aureus</i> NCTC8325 $\Delta pknB$ $\Delta \Phi 13$	NCTC8325 $\Delta pknB$ that had lost the phage 13	26
<i>S. aureus</i> NCTC8325 Δspa Δsbi	<i>spa sbi</i> mutant	23
<i>S. aureus</i> NCTC8325-4	Prophage cured and restriction-deficient derivative of NCTC 8325	27
<i>S. aureus</i> Mu50	Hospital-acquired vancomycin resistant isolate	24
<i>S. aureus</i> MW2	Community-acquired MRSA isolate	28
<i>S. aureus</i> COL	Early hospital-acquired MRSA isolate	29
<i>S. aureus</i> MRSA252	Hospital-acquired MRSA isolate	30
<i>S. aureus</i> MSSA476	Community-acquired methicillin sensitive isolate	30
<i>S. aureus</i> isolates A-J and L-Y	Community- and hospital-acquired clinical isolates collected during a 4.5-year period in the UMCG from 19 patients with different clinical symptoms (for detailed strain descriptions see reference)	31
<i>S. haemolyticus</i>	Opportunistic pathogen clinical strain from UMCG	This study
<i>S. hominis</i>	Human commensal strain obtained from UMCG	This study
<i>E. coli</i> Rosetta gami (DE3)	DE3 lysogen contains T7 polymerase upon IPTG induction.	(Novagen)
pLysS		
<i>L. lactis</i> PA1001	MG1363 <i>pepN::nisRK</i> , Δcma $\Delta htra$	32
Plasmids		
pNG4210	Cm ^R , containing P _{nisaA} , SS _{usp45} , <i>Bam</i> HI/ <i>Eco</i> RI- <i>Xba</i> I/ <i>Not</i> I cloning sites, and <i>his</i> ₆	33
pNG4210:: <i>scn</i>	pNG4210 containing <i>scn</i> with C-terminal <i>his</i> ₆	This study
pNG4210:: <i>chp</i>	pNG4210 containing <i>chp</i> with C-terminal <i>his</i> ₆	This study
prSETB:: <i>scn/orfD</i>	Vectors for expression of chimeric SCIN/OrfD fusions	20

Cm^R chloramphenicol resistance gene, P_{T7} IPTG inducible T7-promoter, P_{nisaA} nisin-inducible promoter, *his*₆ 6x histidine tag, SS_{usp45} signal sequence of *usp45*, MCS multiple cloning site

Table 2. Primers used for detection or cloning of *scn* and *chp* genes

Primer	5' → 3' nucleotide sequence ^a	R.E.
Scn F	ATATGGATCCACAAGCTTGCCAACATCGAATGAATATC	<i>Bam</i> HI
Scn R	ATATGCGGCCGCATATTTACTTTTGTAGTCTCGTCAATTTTC	<i>Not</i> I
Chp F	ATATGGATCCTTTACTTTTGAACCGTTTCCTACAAATG	<i>Bam</i> HI
Chp R	ATATGCGGCCGCATATTCATATTCATTAGTTTTTC	<i>Not</i> I
Scin-up	AGTCTTTTGAAGGAGC	
Scin-low	GTTTATAGCATCACCCTAGTA	

^a restriction enzyme sites are underlined in the nucleotide sequences.

Detection of SCIN bound to whole cells of *S. aureus*

S. aureus isolates were grown overnight in TSB, diluted 1:100 in fresh medium and cultured until the mid-exponential growth phase (OD₆₀₀ ~0.5). Next, the cells were coated with complement by adding serum (end concentration 20%) and incubation was continued for 30 min. After this incubation, the bacteria were washed with phosphate-buffered saline (PBS). High-binding ELISA plates for fluorescence measurements (Greiner Bio-one) were coated with 5×10^6 colony forming units (CFU)

per well in PBS for 18 h at 4°C. Plates were blocked with 4% BSA in PBS with 0.05% Tween-20 (PBST). Surface-bound IgG Fc-binding proteins of *S. aureus* (i.e. Spa and Sbi) were saturated with 100 µg/mL normal rabbit immunoglobulin fraction (DAKO) in PBST containing 1% BSA. The humAb 6D4 was labelled with IRDye 800CW (LI-COR Biosciences, Bad Homburg, Germany) by incubation for 2 hours with 20 µg of IRDye 800CW per mg of protein in PBS (pH 8.5). The mix was desalted following the manufacturer's instructions with a PD minitrapp G-25 desalting column (GE Healthcare, Germany). The resulting 6D4-800CW was stored in the dark at 4°C. To quantify the binding of 6D4-800CW to serum-incubated whole cells with added SCIN, the plates were incubated with 300 ng/mL 6D4-800CW in PBS for 30 min, washed thrice with PBS and scanned with the Odyssey infrared imaging system (LiCor Biosciences) for fluorescence at 800 nm.

Fluorescence microscopy

Overnight cultures in TSB were diluted to an OD₆₀₀ of 10. Untreated samples were taken from the overnight culture. Convertase-covered cell samples were obtained as described above. Cells were collected by centrifugation at 14,000 rpm for 2 min and washed with PBS. The washed cells were incubated with the 6D4- 800CW (3000 ng/mL in PBS) for 30 min. After the incubation, the cells were collected by centrifugation at 14,000 rpm for 2 min and washed with PBS. Next, cells were spotted on a glass slide for microscopy, and a cover slip was mounted and sealed. Fluorescence microscopy was performed using a Leica DM5500B epifluorescence microscope equipped with an 800 nm filter block. Images were captured with a Leica DFC365FX camera using a 63x objective (Leica Microsystems BV, The Netherlands).

Determination of C3b deposition on S. aureus cells

Cells of *S. aureus* Newman $\Delta spa \Delta sbi$ were collected as described above, and 5×10^7 CFU/ml were incubated with 5% pooled normal human serum in HBS *plus* 5 mM CaCl₂, 2.5 mM MgCl₂ and 0.1% human serum albumin for 30 min at 37°C while shaken at 700 rpm. Different concentrations SCIN (0-4 µg/ml) were preincubated with the purified humAb 6D4 (10 µg/ml), with the control human anti-DNP IgG1 (10 µg/ml, Genmab, Utrecht), or with HBS buffer for 10 min at room temperature prior to mixing with the serum. Bacteria were washed by centrifugation and incubated with 1 µg/ml anti-C3b mAb (Quidel Corp.) for 30 min at 4°C followed by APC-labeled Goat-anti-Mouse-Ig (BD Biosciences). Samples were fixed with 1% paraformaldehyde (Polysciences) and analysed on a FACSVerser flow cytometer (BD Biosciences). Data are expressed relative to the mean fluorescence value of bacteria incubated in serum only³⁵.

The alternative pathway hemolytic assay

Washed rabbit erythrocytes at 1×10^8 c/ml (Biotrading) were incubated with 5% pooled normal human serum in HBS *plus* 10 mM MgCl₂ and 10 mM EGTA for 60 min at 37°C while shaken at 600 rpm. Different concentrations SCIN were preincubated with purified humAb 6D4 (10 µg/ml), with the control human anti- DNP IgG1 (10 µg/ml, Genmab, Utrecht), or with HBS-buffer *plus* 10 mM MgCl₂ and 10 mM EGTA for 10 min at room temperature before mixing with serum. Erythrocytes were pelleted and the absorbance of supernatants at 450 nm was measured. Data are expressed relative to the mean value measured for erythrocytes incubated with serum only, which was set to 1³⁶.

Results

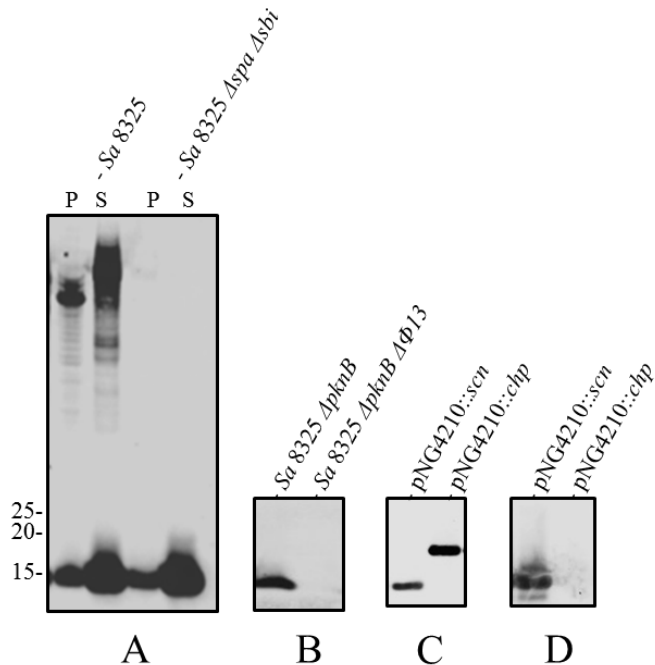
Identification of a human monoclonal antibody that targets the staphylococcal complement inhibitor SCIN

The humAb 6D4 was identified from a random screen of B-cells producing antibodies that bind to whole cells of *S. aureus*. Consequently, the actual target of 6D4 was initially not known. To identify the antigen recognized by 6D4, immunoprecipitation experiments were performed. However, the subsequent Mass Spectrometric analysis of precipitated proteins yielded no conclusive identification of the respective antigen (not shown). As an alternative approach towards target identification, we performed a Western blotting analysis on cells and growth medium fractions of different *S. aureus* isolates. As expected, 6D4 bound to the immunoglobulin-binding proteins Spa (also known as protein A) and Sbi (Fig. 1A). In addition, 6D4 was found to bind a protein of 10-15 kDa that was present both in the cell and growth medium fractions of *S. aureus* NCTC8325, its derivative NCTC8325 ($\Delta spa\Delta sbi$) and NCTC8325 ($\Delta pknB$) (Fig. 1, A and B). The respective signal was however absent from samples of *S. aureus* NCTC8325 ($\Delta pknB\Delta\Phi13$) (Fig. 1B) and *S. aureus* SH1000 (not shown). The latter strains both lack the phage 13 ($\Phi13$)³⁷. This suggested that the antigen recognized by 6D4 was most likely an exported protein of 10-15 kDa encoded by $\Phi13$. Indeed, $\Phi13$ encodes two proteins, SCIN (13 kDa) and the Chemotaxis Inhibitory Protein of *S. aureus* (CHIPS; 17 kDa), which are known to be exported from the cytoplasm to the extracellular milieu.

To test whether 6D4 binds to SCIN or CHIPS, the respective genes were cloned and expressed with a His-tag in *Lactococcus lactis* strain PA1001. As shown by Western blotting with anti His-tag antibodies both SCIN and CHIPS were expressed and secreted by *L. lactis* upon induction with nisin (Fig. 1C). Importantly, the humAb 6D4 was found to bind specifically to SCIN (Fig. 1D). We considered this an important observation as SCIN is a potent inhibitor of the human complement system^{35,36,38}.

Figure 1. Identification of SCIN as target of humAb 6D4

Western blot analysis using humAb 6D4 on proteins from cell pellet (P) and growth medium fractions (supernatant; S) of the *S. aureus* (*Sa*) strains NCTC8325 and NCTC8325 $\Delta spa\Delta sbi$ (A), and the growth medium fractions of strains NCTC8325 $\Delta pknB$ and NCTC8325 $\Delta pknB \Delta\Phi13$ (B). Western blot analysis of the growth medium fractions of *L. lactis* pNG4210::scn or pNG4210::chips secreting the SCIN or CHIPS proteins, respectively, using anti-His-tag antibodies (C), or humAb 6D4 (D). Molecular weights (kDa) of marker proteins are indicated next to panel A.



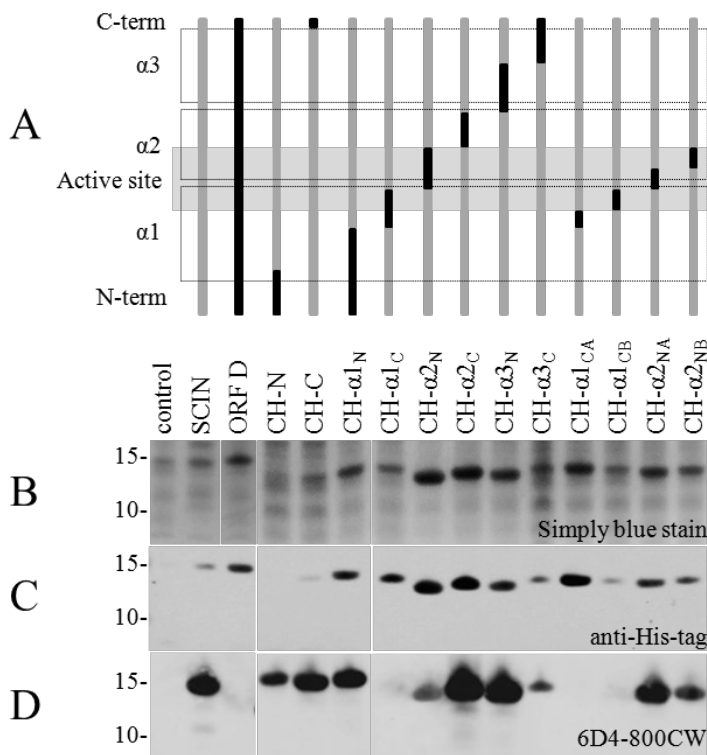


Figure 2. HumAb 6D4 binds to the C-terminal part of the first α -helix of SCIN

Proteins from *E. coli* Rosetta Gami expressing SCIN-OrfD chimera were separated using SDS-PAGE. The expressed chimera of SCIN and OrfD are schematically presented (A). The three helices ($\alpha1$, $\alpha2$ and $\alpha3$) and the active site region of SCIN (in grey shading) are indicated. SCIN residues (grey) were exchanged with corresponding residues from OrfD (black). Exchanged residues (in parentheses) are: CH-N (1-13), CH-C (83-85), CH- $\alpha1$ N (1-25), CH- $\alpha1$ C (26-36), CH- $\alpha2$ N (37-48), CH- $\alpha2$ C (49-58), CH- $\alpha3$ N (59-72), CH- $\alpha3$ C (73-86), CH- $\alpha1$ CA (26-30), CH- $\alpha1$ CB (31-36), CH- $\alpha2$ NA (37-42), and CH- $\alpha2$ NB (43-48). Gels were stained with simply blue to verify protein production (B), and the produced proteins were specifically detected by immunoblotting with an anti-His-antibody (C) or the humAb 6D4-800CW (D). The positions of molecular weight marker proteins (kDa) are shown next to the gel and Western blot images.

HumAb 6D4 binds to the active site of SCIN

To identify the specific SCIN epitope recognized by 6D4, we applied a set of previously constructed *Escherichia coli* Rosetta gami strains expressing IPTG-inducible His-tagged chimera of SCIN and its *S. aureus* homologue OrfD²⁰. The structure of these chimera is schematically represented in Fig. 2A, showing the relative positions of the three α -helices ($\alpha1$, $\alpha2$, and $\alpha3$), the N- and C-termini, and the active site of SCIN. Of note, the OrfD protein has no identified biological activity²⁰, and our humAb 6D4 does not bind to the full-size OrfD (Fig. 2D). All SCIN-OrfD fusion proteins were expressed upon IPTG induction, as shown by SDS-PAGE and Simply blue straining (Fig. 2B) or by immunodetection with anti-His tag antibodies (Fig. 2C), and all detected fusion proteins were of the expected size (Fig. 2, B and C). To assess the binding of 6D4 to the different SCIN-OrfD chimera, this humAb was labelled with the near-infrared fluorophore IRDye 800CW. In Western blotting analyses, the resulting 6D4-800CW facilitated the direct detection of SCIN at 800 nm equally well as the indirect detection of bound 6D4 with a secondary IRDye 800CW-labelled antibody at 800 nm

(results not shown). As shown in Figure 2D, 6D4-800CW bound to most SCIN-OrfD chimera. However, the 6D4-800CW did not bind the CH- α 1-CA fusion, while the CH- α 1-C and CH- α 1-CB fusions were barely bound (Fig. 2D). These findings imply that the epitope recognized by 6D4 is located within the C-terminal half of the first α -helix of the SCIN protein, within amino acid residues 26 to 36. Importantly, these residues overlap with the active site of the SCIN protein²⁰.

HumAb 6D4 specifically binds the *S. aureus* SCIN protein

To verify the specificity of 6D4 for *S. aureus*, we performed a BLAST analysis using the NCBI protein database to identify other bacteria containing SCIN-encoding genes. This showed that the presence of SCIN was restricted to *S. aureus*, and that proteins with limited sequence similarity to SCIN were encoded by the genomes of only few other *Staphylococcus* species, including *S. argenteus* (61% identity from 89% query cover, GenBank: CDR22445.1), *S. hominis* (53% identity from 73% query cover, GenBank: EEK11996.1) and *S. haemolyticus* (57% identity from 74% query cover, GenBank: CPM70056.1). In none of these SCIN homologues was the epitope recognized by 6D4 (i.e. residues 26 to 36) fully conserved. This was confirmed by Western blotting analyses, where 6D4-800CW showed no binding to proteins from *S. hominis* or *S. haemolyticus*, while clear binding to the SCIN proteins of different sequenced *S. aureus* strains was detected (Fig. 3A). Of note, our BLAST analysis indicated that *S. aureus* COL does not contain the *scn* gene encoding SCIN and, consistent with this finding, 6D4-800CW did not bind to any protein of *S. aureus* COL (Fig. 3A).

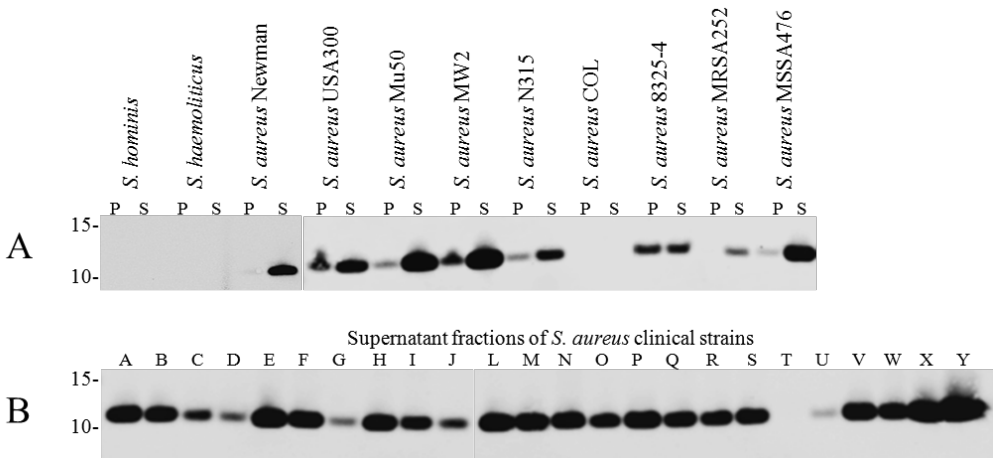


Figure 3. Binding of the humAb 6D4 to SCIN produced by different laboratory strains and clinical isolates of *S. aureus*

Western blotting analysis using humAb 6D4-800CW to detect SCIN in the cell pellet (P) or growth medium (S) fractions of *S. hominis*, *S. haemolyticus* and the *S. aureus* strains Newman, USA300, Mu50, MW2, N315, COL, NCTC8325-4, MRSA252 and MSSA476 (A), or in the growth medium fractions (supernatant) of 24 clinical *S. aureus* isolates named A-J and L-Y (B). Molecular weights (kDa) of marker proteins are indicated to the left of panels A and B. Loading of comparable amounts of proteins was confirmed by Simply Blue staining (not shown).

SCIN is detectable in most clinical *S. aureus* isolates

To explore the production of SCIN by clinical isolates of *S. aureus*, this was assessed with 6D4-800CW in a set of 24 clinical *S. aureus* isolates from the University Medical Center Groningen of which 22 were previously shown by PCR to carry the *scn* gene³¹. Intriguingly, Western blotting with 6D4-800CW revealed the presence of SCIN in 23 of the 24 tested isolates (Fig. 3B), including isolate

G which had tested negative for *scn* in the previous PCR analysis. In contrast, isolate T which had also tested negative for *scn* in the previous PCR also tested negative in the Western blotting with 6D4-800CW. A renewed PCR using *scn*-specific primers showed that the *scn* gene was indeed present in isolate G (data not shown), which is consistent with the detection of SCIN with 6D4-800CW in this isolate. Altogether, these results show that humAb 6D4 labelled with IRDye 800CW can be applied for the specific identification of clinical *S. aureus* isolates expressing SCIN.

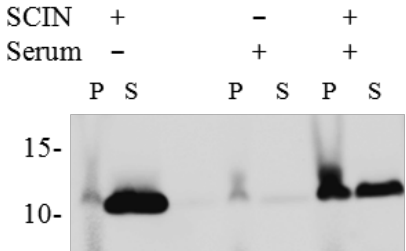
Serum incubation increases binding of SCIN to *S. aureus* cells

The *S. aureus* SCIN protein specifically inhibits the human complement system, one of the most important components of the innate immune system^{20,39-42}. This is achieved through the binding of SCIN to the C3b moiety of human C3 convertases on the bacterial surface, leading to their stabilization in a catalytically inactive form and preventing enhanced conversion of C3 into C3b as part of the so-called 'alternative pathway' in innate immunity. In addition, SCIN promotes the formation of inactive convertase dimers that preclude C3b binding by the complement receptor of phagocytic cells^{40,43}. Because the C3 convertases are key initiators in the complement activation cascades, effector functions such as C3b-mediated phagocytosis and C5a-mediated cell recruitment are effectively prevented by SCIN^{20,35,36,40-43}.

From the Western blotting analyses shown in Figures 1 and 3, it was evident that SCIN is mostly detectable in growth medium fractions, and only to minor extent in the cell fractions when cells are grown in Tryptic Soy Broth (TSB). The latter is consistent with the previously documented finding that SCIN binds to the C3 convertases, which are formed on the *S. aureus* cell wall after initial C3b deposition³⁹. Therefore, we hypothesized that SCIN is likely more abundant in the cell fraction when cell wall-attached C3b is present. To verify this idea, *S. aureus* Newman $\Delta spa\Delta sbi$ cells were covered with C3b through incubation in human sera and, subsequently, these cells were incubated in the presence or absence of added SCIN. As reflected by 6D4-800CW binding upon Western blotting, cells not incubated in serum displayed low levels of SCIN, whereas the respective supernatant fractions yielded a high signal due to the presence of SCIN (Fig. 4). Similarly, the serum-incubated samples without added SCIN showed a low signal in both the cell- and the respective supernatant fractions. In contrast, the serum-incubated samples with added SCIN showed a high SCIN-specific signal in the cell fraction and a lowered signal in the supernatant fraction (Fig. 4). These results show that the enhanced SCIN binding to the *S. aureus* cell wall due to the deposition of C3b and C3 convertases is readily detectable with the 6D4-800CW humAb.

Figure 4. Binding of SCIN to *S. aureus* cells increases upon incubation in serum

Western blotting analysis of *S. aureus* Newman $\Delta spa\Delta sbi$ cells collected by centrifugation (P) and growth medium fractions (S) using 6D4-800CW. The presence or absence of C3 convertases due to serum incubation, and the addition or absence of SCIN are indicated with + or -, respectively.



A plate assay was used to assess whether whole *S. aureus* cells could be detected after incubation with human sera using 6D4-800CW. Indeed, 6D4-800CW was found to bind concentration-dependently to the *S. aureus* clinical isolate P, and the strains USA300, Newman wild-type and Newman $\Delta spa\Delta sbi$ (Fig. 5A). In this assay binding of 6D4 to Spa and Sbi via the Fc-region was

blocked by the addition of unrelated rabbit IgG, and effective blocking was confirmed with a control His-tag-specific rabbit antibody (α -his-tag; Fig. 5A). Importantly, 6D4-800CW allowed the detection of cell-bound SCIN in 19 of 24 clinical *S. aureus* isolates tested (Fig. 5B). Here it is noteworthy that 5 isolates showed no enhanced binding of SCIN, including four *scn*-proficient isolates and the isolate T lacking the *scn* gene. Furthermore, 6D4-800CW allowed detection of cell-bound SCIN for 8 of 9 sequenced *S. aureus* strains, where only the COL strain that lacks the *scn* gene yielded no signal (Fig. 5B). Binding of the α -his-tag control antibody was low for all strains due to blocking with an unrelated rabbit IgG (Fig. 5B).

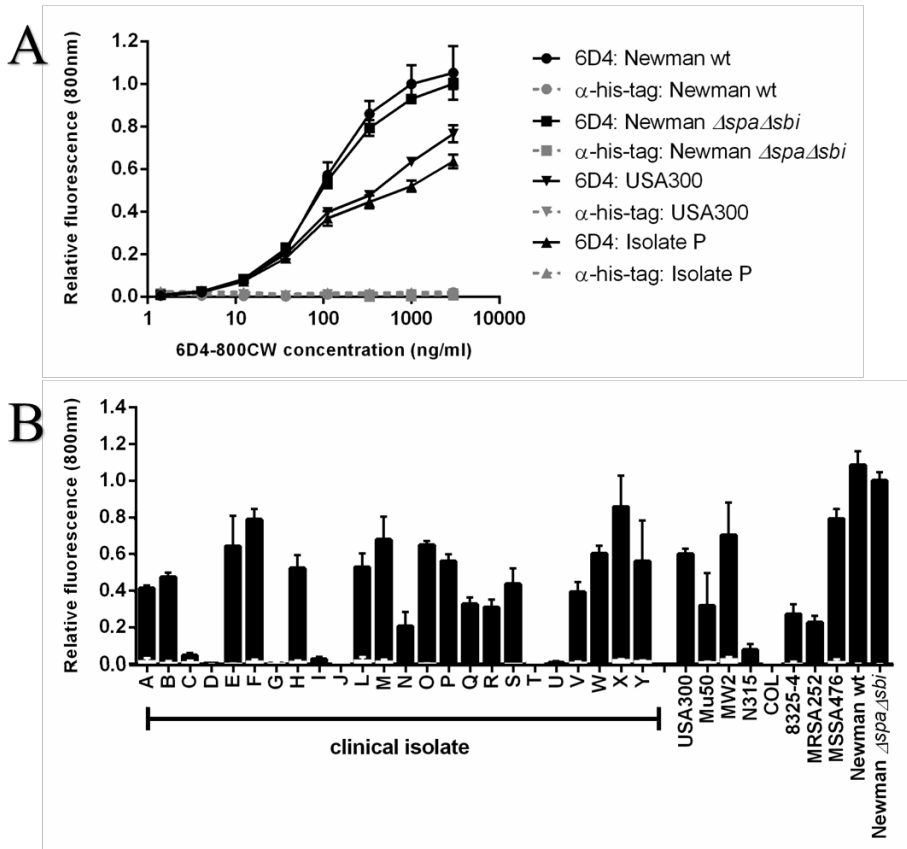


Figure 5. Binding of the humAb 6D4 to whole cells of *S. aureus*

Plates were coated with whole cells of various *S. aureus* clinical isolates or laboratory strains harvested from cultures in the mid-exponential growth phase where the growth medium was supplemented with human serum. 6D4-800CW was used for the detection of cell-bound SCIN, and an α -his-tag antibody was used as a negative control. Fluorescence readings at 800 nm are plotted relative to the binding of 6D4-800CW to *S. aureus* Newman $\Delta spa\Delta sbi$. All measurements were performed in triplicate and the mean \pm standard error (error bars) is shown. (A) concentration-dependent binding of 6D4-800CW to *S. aureus* Newman $\Delta spa\Delta sbi$, Newman wild-type (wt), the clinical *S. aureus* isolate P, or the MRSA strain USA300 is indicated in black symbols; the lack of binding of the α -his-tag control antibody to *S. aureus* Newman $\Delta spa\Delta sbi$, Newman wild-type (wt), isolate P, or USA300 is shown in grey symbols. (B) Binding of 6D4-800CW to *S. aureus* Newman $\Delta spa\Delta sbi$, various clinical *S. aureus* isolates and the sequenced *S. aureus* strains USA300, Mu50, MW2, N315, COL, 8325-4, MRSA252, MSSA476, Newman wild-type (WT) and Newman $\Delta spa\Delta sbi$ is indicated with black bars; binding of 100 ng/mL isotype control antibody IQNPA to the *S. aureus* clinical isolates and sequenced *S. aureus* strains as specified is indicated with white bars.

Direct detection of SCIN bound to the surface of *S. aureus* cells

For direct detection of SCIN bound to the surface of *S. aureus* cells, samples of *S. aureus* Newman $\Delta spa\Delta sbi$ were prepared and spotted onto glass slides for fluorescence microscopy at 800 nm. *S. aureus* cells grown under standard culturing conditions and incubated with 6D4-800CW displayed almost no fluorescence and individual cells could not be distinguished (Fig. 6, A and B). Further, cells incubated in serum, but lacking added SCIN, showed no fluorescent signal at all (Fig. 6, C and D). Importantly however, serum-incubated cells with added SCIN showed a strongly enhanced fluorescent signal at 800 nm (Fig. 6, E and F). Here individual cells were detectable, though it is noteworthy that not all cells appeared to be fluorescently tagged. Taken together, these observations show that *S. aureus* cells incubated with human serum have a high potency for binding of SCIN, most likely due to the deposition of C3b and C3 convertases, which can be detected with IRDye 800CW-labelled 6D4 humAb.

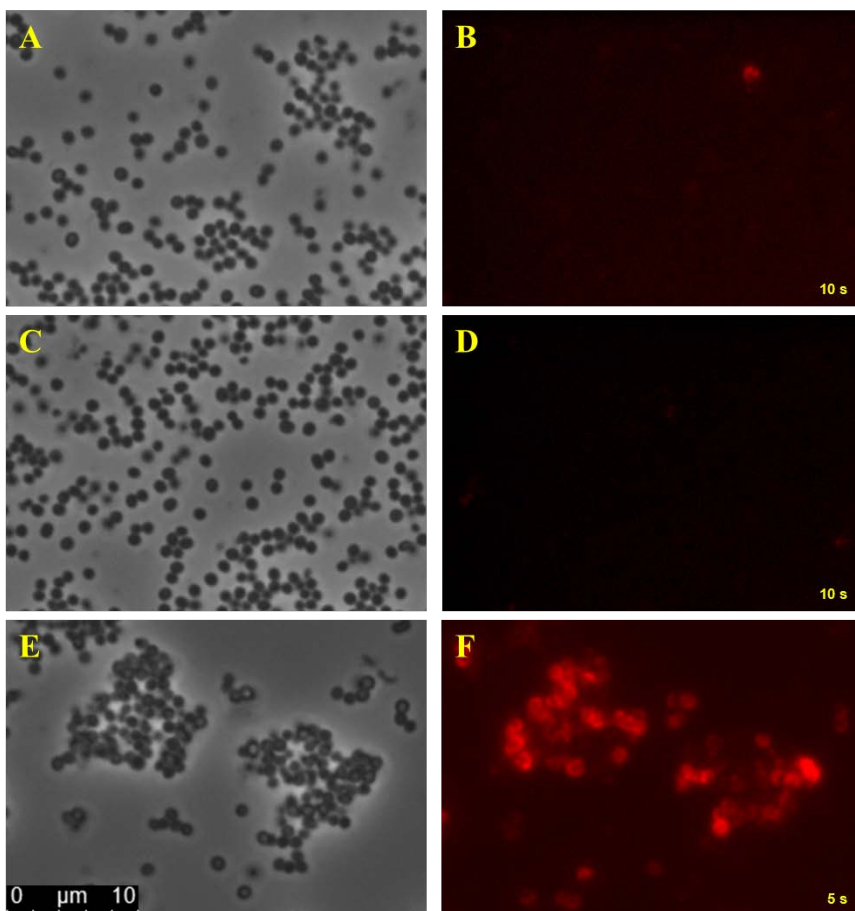


Figure 6. Serum-incubated *S. aureus* cells display elevated levels of SCIN binding

Phase contrast (panels A, C, E) and subsequent fluorescence microscopy at 800 nm (panels B, D, F) of cells of *S. aureus* Newman $\Delta spa\Delta sbi$ collected from an overnight culture. Specifically, the panels show cells from the overnight culture (A, B), cells treated with serum but without the addition of SCIN (C, D), and cells treated with serum and added SCIN (E, F). Cell-bound SCIN was detected using 6D4-800CW.

Impact of 6D4 on SCIN activity

Since the humAb 6D4 binds to the active site of SCIN, we asked the question how this antibody impacts on the deposition of C3b on the *S. aureus* cell surface. To this end, we employed an assay where increasing amounts of SCIN were pretreated with 6D4, prior to mixing with human serum. As controls, the SCIN protein was mock-treated with buffer or a control IgG prior to mixing with serum. Next, *S. aureus* Newman $\Delta spa\Delta sbi$ cells were incubated for 30 min with the serum containing SCIN (with or without 6D4 pretreatment), after which the presence of C3b on the staphylococcal cell surface was measured by flow cytometry. As shown in Figure 7A, in this assay the preincubation of SCIN with humAb 6D4 resulted in a relative deposition of C3b on the *S. aureus* cells close to 1, which represents the maximal C3b deposition upon incubation with serum. In contrast, the C3b deposition was inhibited by SCIN in the absence of 6D4. These findings imply that 6D4 can interfere with the deposition of C3b on the *S. aureus* cells.

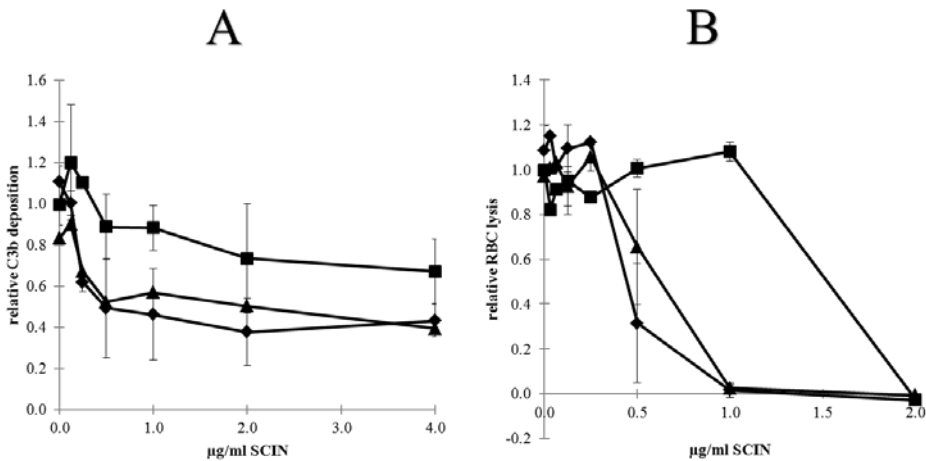


Figure 7. Impact of humAb 6D4 on SCIN activity

(A) C3b deposition on *S. aureus* Newman $\Delta spa\Delta sbi$ cells upon preincubation of SCIN with humAb 6D4 (■). C3b deposition was monitored by flow cytometry. As a negative control, SCIN was preincubated with buffer (◆), or control IgG (▲). Each data point represents the mean \pm standard error (error bars) of three independent experiments. (B) Reduced SCIN-mediated protection of rabbit erythrocytes against lysis by complement upon incubation of SCIN with humAb 6D4 (■). Hemolysis was quantified by pelleting of erythrocytes and subsequent measurement of the absorbance of supernatants at 450 nm. As a control, SCIN was preincubated with buffer (◆), or control IgG (▲). Each data point represents the mean \pm standard error (error bars) of two separate experiments.

An alternative possibility to measure the impact of 6D4 on SCIN activity is provided by the fact that complement causes the lysis of rabbit erythrocytes, and that this hemolysis can be inhibited by SCIN. To assess whether SCIN-mediated inhibition of the alternative pathway's hemolytic activity can be suppressed by 6D4, we pre-treated increasing amounts of SCIN with 6D4, prior to mixing with human serum and erythrocytes. As a negative control, the SCIN protein was either mock-treated with buffer or a control IgG prior to mixing with serum and erythrocytes. Next, the erythrocytes were incubated for 60 min with the human serum containing SCIN (with or without 6D4 pre-treatment), after which the erythrocytes were pelleted and the absorbance of supernatants at 450 nm was measured to assess the erythrocyte lysis. As shown in Figure 7B, the preincubation of SCIN with 6D4

significantly reduced the protective effect of SCIN with respect to erythrocyte lysis, as compared to SCIN preincubated with the control IgG or with buffer. These observations fully support the view that the activity of SCIN can be inhibited by the humAb 6D4.

Discussion

In this study, we show that the humAb 6D4 binds to the first α -helix of the staphylococcal complement inhibitor SCIN, which covers part of this protein's active site domain. Consistent with this finding, 6D4 interferes with the activity of SCIN, as shown through the analysis of C3b deposition on *S. aureus* cells and suppression of the protective effect of SCIN in the alternative pathway-mediated hemolysis of rabbit erythrocytes. Furthermore, we show that 6D4 labelled with the near-infrared fluorophore IRDye 800CW can be readily used to visualize the production and subcellular localization of SCIN by *S. aureus*.

The analysis of publicly available bacterial genome sequences suggests that the *scn* gene is specific for *S. aureus* isolates causing infections in humans. While sequenced *S. hominis* and *S. haemolyticus* strains contain genes with some sequence similarity to the *S. aureus* *scn* gene, the tested *S. hominis* and *S. haemolyticus* strains did not bind humAb 6D4. This underpins the conclusion that this humAb is highly specific for *S. aureus* SCIN, and suggests that it will bind preferentially to isolates associated with infections in humans. Previous studies have reported that SCIN may be present in 90% of all clinical *S. aureus* isolates and that it is expressed in vivo^{20,35,36,38}. Consistent with this view, we observed that, from a panel of 33 tested *S. aureus* isolates, only two did not express SCIN.

SCIN is a potent antigen that evokes high antibody titres in *S. aureus*-colonized individuals^{18,44,45}. Under the in vitro conditions used for culturing *S. aureus* in this study, the clearest SCIN signals were obtained for growth medium fractions, while the signals in the respective *S. aureus* cell fractions were relatively low. On the other hand, our present findings show that SCIN was effectively recruited to the *S. aureus* cell surface when this bacterium was exposed to human serum. This phenomenon was also clearly evident at the single cell level by fluorescence microscopy. The observed redistribution of SCIN is consistent with the fact that SCIN binds to the C3b moiety of C3 convertases upon their deposition on the bacterial cell surface⁴⁶. This puts emphasis on the extensive interactions between *S. aureus* and its human host, which are underestimated under the generally applied in vitro culturing conditions. Indeed this view is confirmed by a previous study showing that *S. aureus* cells bind a variety of human proteins to their cell surface upon incubation in plasma⁴⁷. Of note, when serum-incubated clinical *S. aureus* isolates and lab strains were tested for enhanced binding of SCIN using 6D4-800CW, only 4 out of 34 investigated strains remained undetectable, which suggests that they only bind small amounts of SCIN. Notably, our Western blotting analyses show that these strains produce relatively low amounts of SCIN, which might not be sufficient to distinguish the SCIN-specific signal from the background signal in a whole cell plate reader-based approach. Of note, upon fluorescence microscopy, not all *S. aureus* cells appeared to bind equal amounts of 6D4-800CW, suggesting that there may be cell-to-cell differences in the formation of C3 convertases, the binding of SCIN or the binding of 6D4-800CW.

In conclusion, in the present study we present a humAb that binds to the active site of the *S. aureus* SCIN protein, especially residues 26-36. While the humAb 6D4 does interfere with the activity of SCIN, it seems rather unlikely that it can be applied in antistaphylococcal therapy since SCIN-deficient variants of *S. aureus* can also cause infections. Importantly however, the IRDye 800CW-labelled version of this humAb (i.e. 6D4-800CW) can be applied to specifically detect *S. aureus*

isolates that express SCIN, an important virulence factor that allows *S. aureus* to effectively evade the human complement system. A completely novel finding is that SCIN binding to the staphylococcal cell-surface is substantially enhanced in the presence of human serum. Since SCIN production is associated in particular with *S. aureus* isolates that caused infections in humans, our SCIN-specific antibody may find potential future applications in the identification of *S. aureus* lineages with a high potential for causing infections. This could not only involve diagnostic tests, but also in vivo imaging approaches for which proof-of-principle was recently obtained using vancomycin labelled with the IRDye 800CW^{48–50}.

Acknowledgements

We thank the anonymous patients with EB from the Dutch Epidermolysis Bullosa Registry for blood donations, Martin van der Heide and Tim van der Toorn for technical support and Annette Dreisbach for helpful discussions.

Funding Statement

Part of this research was supported by the Top Institute Pharma projects T4-213. F.R.P. received a scholarship from CONACyT (169643) and was supported in parts by the Graduate School for Medical Sciences of the University of Groningen. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author's Contributions

HH, FRP, KPMvK, JMvD and GB conceived and designed the experiments. HH, FRP, HPJB, KPMvK, GSA and NK performed the experiments. HH, FRP, KPMvK, JMvD and GB analyzed the data. HPJB, KPMvK, HG, JMvD and GB contributed reagents, materials and analysis tools. HH, FRP, KPMvK, JMvD and GB wrote the manuscript. All authors have reviewed and approved the final manuscript.

References

1. Sibbald, M. J. J. B. *et al.* Partially overlapping substrate specificities of staphylococcal group A sortases. *PROTEOMICS* **12**, 3049–3062 (2012).
2. Stefani, S. & Goglio, A. Methicillin-resistant *Staphylococcus aureus*: related infections and antibiotic resistance. *Int. J. Infect. Dis.* **14**, Supplement 4, S19–S22 (2010).
3. Thammavongsa, V., Kim, H. K., Missiakas, D. & Schneewind, O. Staphylococcal manipulation of host immune responses. *Nat. Rev. Microbiol.* **13**, 529–543 (2015).
4. Stryjewski, M. E. & Corey, G. R. Methicillin-Resistant *Staphylococcus aureus*: An Evolving Pathogen. *Clin. Infect. Dis.* **58**, S10–S19 (2014).
5. Cosgrove, S. E. *et al.* The impact of methicillin resistance in *Staphylococcus aureus* bacteremia on patient outcomes: mortality, length of stay, and hospital charges. *Infect. Control Hosp. Epidemiol.* **26**, 166–174 (2005).
6. Hidron, A. I. *et al.* Antimicrobial-Resistant Pathogens Associated With Healthcare-Associated Infections: Annual Summary of Data Reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006–2007. *Infect. Control Hosp. Epidemiol.* **29**, 996–1011 (2008).
7. Moellering, J., Robert C. The Growing Menace of Community-Acquired Methicillin-Resistant *Staphylococcus aureus*. *Ann. Intern. Med.* **144**, 368–370 (2006).
8. Holmes, N. E., Johnson, P. D. R. & Howden, B. P. Relationship between Vancomycin-Resistant *Staphylococcus aureus*, Vancomycin-Intermediate *S. aureus*, High Vancomycin MIC, and Outcome in Serious *S. aureus* Infections. *J. Clin. Microbiol.* **50**, 2548–2552 (2012).
9. Rupp, M. E. *et al.* Phase II, Randomized, Multicenter, Double-Blind, Placebo-Controlled Trial of a Polyclonal Anti-*Staphylococcus aureus* Capsular Polysaccharide Immune Globulin in Treatment of *Staphylococcus aureus* Bacteremia. *Antimicrob. Agents Chemother.* **51**, 4249–4254 (2007).
10. Jansen, K. U., Girgenti, D. Q., Scully, I. L. & Anderson, A. S. Vaccine review: ‘*Staphylococcus aureus* vaccines: Problems and prospects’. *Vaccine* **31**, 2723–2730 (2013).
11. Sause, W. E., Buckley, P. T., Strohl, W. R., Lynch, A. S. & Torres, V. J. Antibody-Based Biologics and Their Promise to Combat *Staphylococcus aureus* Infections. *Trends Pharmacol. Sci.* **37**, 231–241 (2016).
12. Scott, A. M., Wolchok, J. D. & Old, L. J. Antibody therapy of cancer. *Nat. Rev. Cancer* **12**, 278–287 (2012).
13. van den Berg, S. *et al.* A human monoclonal antibody targeting the conserved staphylococcal antigen IsaA protects mice against *Staphylococcus aureus* bacteremia. *Int. J. Med. Microbiol.* **305**, 55–64 (2015).
14. Lorenz, U. *et al.* Functional Antibodies Targeting IsaA of *Staphylococcus aureus* Augment Host Immune Response and Open New Perspectives for Antibacterial Therapy. *Antimicrob. Agents Chemother.* **55**, 165–173 (2011).
15. Oesterreich, B. *et al.* Characterization of the biological anti-staphylococcal functionality of hUK-66 IgG1, a humanized monoclonal antibody as substantial component for an immunotherapeutic approach. *Hum. Vaccines Immunother.* **10**, 926–937 (2014).
16. van der Kooi-Pol, M. M., Duipmans, J. C., Jonkman, M. F. & van Dijk, J. M. Host–pathogen interactions in epidermolysis bullosa patients colonized with *Staphylococcus aureus*. *Int. J. Med. Microbiol.* **304**, 195–203 (2014).

17. van der Kooi-Pol, M. M. *et al.* High genetic diversity of *Staphylococcus aureus* strains colonizing patients with epidermolysis bullosa. *Exp. Dermatol.* **21**, 463–466 (2012).
18. van der Kooi-Pol, M. M. *et al.* High Anti-Staphylococcal Antibody Titers in Patients with Epidermolysis Bullosa Relate to Long-Term Colonization with Alternating Types of *Staphylococcus aureus*. *J. Invest. Dermatol.* **133**, 847–850 (2013).
19. Swierstra, J. *et al.* IgG4 Subclass-Specific Responses to *Staphylococcus aureus* Antigens Shed New Light on Host-Pathogen Interaction. *Infect. Immun.* **83**, 492–501 (2015).
20. Rooijackers, S. H. M. *et al.* Staphylococcal Complement Inhibitor: Structure and Active Sites. *J. Immunol.* **179**, 2989–2998 (2007).
21. Duthie, E. S. & Lorenz, L. L. Staphylococcal Coagulase: Mode of Action and Antigenicity. *J. Gen. Microbiol.* **6**, 95–107 (1952).
22. Sibbald, M. J. J. B. *et al.* Synthetic Effects of secG and secY2 Mutations on Exoproteome Biogenesis in *Staphylococcus aureus*. *J. Bacteriol.* **192**, 3788–3800 (2010).
23. McDougal, L. K. *et al.* Pulsed-Field Gel Electrophoresis Typing of Oxacillin-Resistant *Staphylococcus aureus* Isolates from the United States: Establishing a National Database. *J. Clin. Microbiol.* **41**, 5113–5120 (2003).
24. Kuroda, M. *et al.* Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. *The Lancet* **357**, 1225–1240 (2001).
25. Donat, S. *et al.* Transcriptome and Functional Analysis of the Eukaryotic-Type Serine/Threonine Kinase PknB in *Staphylococcus aureus*. *J. Bacteriol.* **191**, 4056–4069 (2009).
26. Miller, M. Mapping the interactions between *Staphylococcus aureus* and host immune cells. (s.n., 2012).
27. Kreiswirth, B. N. *et al.* The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. *Nature* **305**, 709–712 (1983).
28. Baba, T. *et al.* Genome and virulence determinants of high virulence community-acquired MRSA. *The Lancet* **359**, 1819–1827 (2002).
29. Gill, S. R. *et al.* Insights on Evolution of Virulence and Resistance from the Complete Genome Analysis of an Early Methicillin-Resistant *Staphylococcus aureus* Strain and a Biofilm-Producing Methicillin-Resistant *Staphylococcus epidermidis* Strain. *J. Bacteriol.* **187**, 2426–2438 (2005).
30. Holden, M. T. G. *et al.* Complete genomes of two clinical *Staphylococcus aureus* strains: Evidence for the rapid evolution of virulence and drug resistance. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 9786–9791 (2004).
31. Ziebandt, A.-K. *et al.* Proteomics uncovers extreme heterogeneity in the *Staphylococcus aureus* exoproteome due to genomic plasticity and variant gene regulation. *PROTEOMICS* **10**, 1634–1644 (2010).
32. Bosma, T. *et al.* Novel Surface Display System for Proteins on Non-Genetically Modified Gram-Positive Bacteria. *Appl. Environ. Microbiol.* **72**, 880–889 (2006).
33. Neef, J. *et al.* Versatile vector suite for the extracytoplasmic production and purification of heterologous His-tagged proteins in *Lactococcus lactis*. *Appl. Microbiol. Biotechnol.* 1–12 (2015). doi:10.1007/s00253-015-6778-8
34. Leenhouts, K. & Venema, G. Lactococcal plasmid vectors. *Plasmids Pract. Approach Oxf. Univ. Press Oxf. U. K.* 65–94 (1993).

35. Rooijakkers, S. H. M. *et al.* Immune evasion by a staphylococcal complement inhibitor that acts on C3 convertases. *Nat. Immunol.* **6**, 920–927 (2005).
36. Jongerius, I. *et al.* Staphylococcal complement evasion by various convertase-blocking molecules. *J. Exp. Med.* **204**, 2461–2471 (2007).
37. O'Neill, A. J. *Staphylococcus aureus* SH1000 and 8325-4: comparative genome sequences of key laboratory strains in staphylococcal research. *Lett. Appl. Microbiol.* **51**, 358–361 (2010).
38. Wamel, W. J. B. van, Rooijakkers, S. H. M., Ruyken, M., Kessel, K. P. M. van & Strijp, J. A. G. van. The Innate Immune Modulators Staphylococcal Complement Inhibitor and Chemotaxis Inhibitory Protein of *Staphylococcus aureus* Are Located on β -Hemolysin-Converting Bacteriophages. *J. Bacteriol.* **188**, 1310–1315 (2006).
39. Serruto, D., Rappuoli, R., Scarselli, M., Gros, P. & van Strijp, J. A. G. Molecular mechanisms of complement evasion: learning from staphylococci and meningococci. *Nat. Rev. Microbiol.* **8**, 393–399 (2010).
40. Jongerius, I. *et al.* Staphylococcal Complement Inhibitor Modulates Phagocyte Responses by Dimerization of Convertases. *J. Immunol.* **184**, 420–425 (2010).
41. Garcia, B. L., Ramyar, K. X., Ricklin, D., Lambris, J. D. & Geisbrecht, B. V. Advances in Understanding the Structure, Function, and Mechanism of the SCIN and Efb Families of Staphylococcal Immune Evasion Proteins. *Adv. Exp. Med. Biol.* **946**, 113–133 (2012).
42. Garcia, B. L. *et al.* A Structurally Dynamic N-terminal Helix Is a Key Functional Determinant in Staphylococcal Complement Inhibitor (SCIN) Proteins. *J. Biol. Chem.* **288**, 2870–2881 (2013).
43. Rooijakkers, S. H. M. *et al.* Structural and functional implications of the alternative complement pathway C3 convertase stabilized by a staphylococcal inhibitor. *Nat. Immunol.* **10**, 721–727 (2009).
44. van den Berg, S. *et al.* A multiplex assay for the quantification of antibody responses in *Staphylococcus aureus* infections in mice. *J. Immunol. Methods* **365**, 142–148 (2011).
45. Glasner, C. *et al.* Low anti-staphylococcal IgG responses in granulomatosis with polyangiitis patients despite long-term *Staphylococcus aureus* exposure. *Sci. Rep.* **5**, (2015).
46. Merle, N. S., Church, S. E., Fremeaux-Bacchi, V. & Roumenina, L. T. Complement System Part I – Molecular Mechanisms of Activation and Regulation. *Front. Immunol.* **6**, (2015).
47. Dreisbach, A. *et al.* Surface shaving as a versatile tool to profile global interactions between human serum proteins and the *Staphylococcus aureus* cell surface. *PROTEOMICS* **11**, 2921–2930 (2011).
48. Oosten, M. van *et al.* Real-time in vivo imaging of invasive- and biomaterial-associated bacterial infections using fluorescently labelled vancomycin. *Nat. Commun.* **4**, 2584 (2013).
49. Oosten, M. van *et al.* Targeted imaging of bacterial infections: advances, hurdles and hopes. *FEMS Microbiol. Rev.* **39**, 892–916 (2015).
50. Heuker, M. *et al.* Preclinical studies and prospective clinical applications for bacteria-targeted imaging: the future is bright. *Clin. Transl. Imaging* **4**, 253–264 (2016).